Stable Prostacyclin Analogue Preventing Microcirculatory Derangement in Experimental Cerebral Ischemia in Cats

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We evaluated the effect of a stable synthetic prostacyclin analogue, TRK-100, on the microcirculatory derangement occurring in feline pial vessels with endothelial damage after middle cerebral artery occlusion. Fifteen adult cats were divided into an untreated group (Group 1, n=8) and a treated group (Group 2, n=7). Thirty minutes after 10 minutes of ultraviolet irradiation, which selectively damaged endothelium in the pial vessels, the middle cerebral artery was occluded in both groups and maintained for 30 minutes. In Group 2, 50 ng/kg/min TRK-100 was continuously infused intravenously following ultraviolet irradiation. In both the pial arteries and veins, platelet aggregate adhesion to the endothelium with subsequent thrombus formation was significantly (p<0.01 and p<0.05, respectively) inhibited during middle cerebral artery occlusion in Group 2 compared with Group 1. Similarly, blood flow stasis in the pial veins was effectively prevented in Group 2 during occlusion. Furthermore, the pial artery diameter returned to the control level during the late period of occlusion, whereas in Group 1 the pial artery remained constricted. Our data suggest that TRK-100 can prevent microcirculatory derangement in the acute stage of ischemic stroke.

Recent, we developed an animal model with selective endothelial damage in the pial vessels using an irradiation technique with ultraviolet (UV) rays.1,2 We found that the interaction of platelets with the damaged endothelium is important in the progressive disturbance of the pial microcirculation following occlusion of a major cerebral artery.1,2

In the past decade, numerous therapeutic attempts have been made to find effective measures for interrupting pathologic platelet–vessel wall interactions3,4 in which the metabolism of arachidonic acid and prostaglandins is essentially involved.5–7 Prostacyclin, a naturally occurring prostaglandin released from endothelial cells, is the most powerful endogenous inhibitor of platelet aggregation yet discovered.8 Several studies have therefore been undertaken to test the usefulness of prostacyclin in the treatment of ischemic stroke in either a clinical or experimental setting, although the results, especially in clinical studies,9–19 have not been conclusive. One reason for this questionable effect of prostacyclin on ischemic stroke may be its very labile chemical properties (t1/2 at 37° C = 3 minutes),8 so that maintenance of an effective concentration in the circulation seems to be very difficult. Furthermore, prostacyclin is also a potent vasodilator with a marked hypotensive action,8 which may cause hypoperfusion in ischemic brain tissue where autoregulation of cerebral blood flow is frequently perturbed.

In view of the therapeutic potential of prostacyclin and the characterization of its chemical structure, several synthetic prostacyclin analogues have been developed in recent years.20,21 Among them, TRK-100 is chemically very stable and maintains its potent inhibitory character against platelet aggregation for >6 months at 50° C in vitro.22,23 Oral administration of TRK-100 is possible, suggesting a wide clinical application if its effectiveness in ischemic stroke can be demonstrated.

We evaluated the effect of TRK-100 on the microcirculatory derangement occurring in pial vessels with endothelial damage following middle cerebral artery occlusion (MCAO) in cats.

Materials and Methods

We used 15 adult cats of either sex weighing 2.0–4.0 kg. Anesthesia, respiratory control, and place-
TABLE 1. Physiologic Variables in Cats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Artery (µm)</td>
<td>Vein (µm)</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>127.8±6.3</td>
<td>176.1±12.0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>116.1±9.5</td>
<td>200.3±19.9</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Group 1, untreated; Group 2, infusion of 50 ng/kg/min TRK-100.

The experimental setup we employed was as described elsewhere. Briefly, a cranial window made of quartz with a stainless steel frame was screwed into the left parietal region of the calvaria after placing the head of each cat in a head holder. Catheters were inserted into the right femoral artery and vein to record the systemic arterial blood pressure and to infuse TRK-100, respectively.

The protocol of the cranial window were as described elsewhere. Briefly, a cranial window made of quartz with a stainless steel frame was screwed into the left parietal region of the calvaria after placing the head of each cat in a head holder. Catheters were inserted into the right femoral artery and vein to record the systemic arterial blood pressure and to infuse TRK-100, respectively.

The experimental setup we employed was as described elsewhere. Briefly, the pial vessels and platelets were visualized at ×200 magnification on a television (TV) monitor, and the diameters of the vessels of interest were recorded continuously with a multipen recorder. The output of the TV monitor was recorded on a videotape recorder (VO-5850, Sony, Tokyo, Japan) so that the images of the pial vessels could be analyzed later with the image analyzer developed in our laboratory by replaying the tape. Platelet aggregates can be distinguished from the vessel wall and the plasma based on their optical properties using the image analyzer, which converts the analog video image into 512 x 512 digital data and generates an 8-bit (256 unit) gray value for each pixel. A 150-W xenon lamp with a heat-cut filter and a specially designed glass (low-cut) filter that cut wavelengths of <420 nm was used for illuminating the brain surface through a quartz glass fiber. UV irradiation of the pial vessels was carried out with the same system, but without the low-cut filter. The intensity of UV rays on the brain surface was measured with a silicon diode detector (UIT-101, Ushio, Tokyo, Japan) and was adjusted to between 3.0 and 6.0 mW/cm².

In eight cats designated as Group 1, after the brain surface had been irradiated with UV rays for 10 minutes to cause slight damage to the endothelium, the pial vessels were observed under illumination with wavelengths of >420 nm for 30 minutes. The left middle cerebral artery (MCA) was occluded with a Scoville clip for 30 minutes by the transorbital approach, followed by reopening of the occlusion and subsequent observation for an additional 20 minutes. In the remaining seven cats (Group 2), after UV irradiation for 10 minutes, continuous intravenous infusion of 50 ng/kg/min TRK-100 was begun and maintained throughout the remainder of the study, the protocol of which was identical to...
that in Group 1. TRK-100 was dissolved in phosphate-buffered saline to give a concentration of 18 μg/100 ml at pH 7.4.

At the end of the experiments, the observed pial vessels were fixed with topically applied 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer. On termination of this procedure, the vessels were removed and placed in the same fixative for histologic examination under a transmission electron microscope (HU10, Hitachi, Tokyo, Japan).

Platelet aggregation was quantified as the time required for the first visible microaggregate to be produced at the site of endothelial damage but to be flushed away without adhering to the vessel wall after MCAO (appearance of microaggregates), as the time required for the first aggregate to adhere to the vessel wall after MCAO (adhesion to endothelium), and as the time between the initiation of MCAO and total blockade of blood flow by enlarging aggregates in the vessel (stasis). Data are given as the mean± standard error of the mean. The incidence of these phenomena was also counted.

Results

Table 1 shows the mean arterial blood pressure (MABP) and the diameter of the observed pial vessels at the start of the experiments for each group. There was no significant difference in each parameter between the two groups.

Figure 1 illustrates sequential changes in the video images of pial vessels in cats in Group 1. Before MCAO, minimal platelet aggregates were observed in the pial artery, which runs vertically on the image (diameter 150 μm), whereas the pial vein on the right side of the image showed a longitudinally extended thrombus on the endothelium caused by the UV irradiation. After MCAO, progressive development of a number of platelet thrombi was noted in the large pial artery as well as in the small arteries, resulting in stasis of blood flow within these vessels. The pial veins also revealed similar formation of thrombi after MCAO, with subsequent stasis of blood flow. Blood flow was not restored after reopening of the MCA in either the pial arteries or veins.

Figure 2 illustrates sequential changes in the video images of pial vessels in cats in Group 2. Thirty minutes after initiation of the TRK-100 infusion, both the large pial artery (left side of the image, diameter 150 μm) and vein (right side of the image, diameter 95 μm) showed mild dilatation. Following MCAO, the pial artery constricted markedly, and numerous platelet microaggregates were produced at the site of endothelial damage caused by the UV rays, but the aggregates were continuously swept downstream. In the pial vein, occlusion...
of the MCA resulted in a marked decrease in the velocity of blood flow, accompanied by plasma gaps in the blood stream, as well as the formation of numerous platelet microaggregates at the site of the damaged endothelium. In spite of such rheologic alterations, neither adhesion of platelet aggregates nor formation of thrombi was observed in the pial artery and vein. After reopening of the MCA, blood flow returned to almost normal.

Figure 3 shows an electron micrograph of the pial artery from a cat in Group 1. The intraluminal space of the vessel was tightly packed with platelet aggregates, in which a majority of the platelets were degranulated and/or were forming pseudopodia. A number of platelets adhered to the endothelium. Mild vacuolation of the endothelium was observed, but the muscle layer was intact.

Figure 4 shows an electron micrograph of the pial artery from a cat in Group 2. Despite mild vacuolation of the endothelium comparable to that in cats in Group 1, there were no visible platelet aggregates adhering to the endothelium.

Figure 5 illustrates the changes in diameter of the pial arteries and in MABP in Group 1 cats through-
out the experiment after termination of the UV irradiation. There was almost no change in diameter for 30 minutes before MCAO. However, immediately after MCAO, the artery constricted significantly (6.3 ± 1.4% of control diameter, \( p < 0.05 \)) and remained constricted until reopening of the MCAO. After reopening, the artery exhibited a moderate and continuous dilatation accompanied by a gradual decline of MABP.

Figure 6 illustrates the changes in diameter of the pial arteries and in MABP in Group 2 cats after the start of TRK-100 infusion. The pial arteries dilatated progressively for 15 minutes after the initiation of TRK-100 infusion, at which time the diameter had increased significantly by 8.0 ± 2.0% over the preinfusion level (\( p < 0.01 \)). The dilatation continued until MCAO, when the pial arteries constricted markedly. Four minutes after initiation of MCAO, maximum constriction, 6.1 ± 3.0% of the control level, was noted. Thereafter, the pial arteries gradually recovered their diameter, and 15 minutes after the initiation of MCAO, the diameter almost returned to the preinfusion level, which was maintained until reopening of the MCAO. When the clip on the MCA was removed, the pial arteries dilatated immediately and significantly. On the other hand, MABP did not change significantly throughout the study.

Compared with Group 1, the pial arteries were dilatated significantly 10, 15, and 30 minutes after initiation of the TRK-100 infusion (\( p < 0.05 \), 0.02, and 0.02, respectively). During the late phase of MCAO, Group 2 pial arteries recovered (\( p < 0.01 \) compared with diameter 4 minutes after MCAO), which contrasted with the sustained constriction observed in Group 1.
Table 2 summarizes the frequency of and time to appearance of microaggregates, frequency of and time to adhesion of platelet aggregates to the endothelium, frequency of and time to stasis of blood flow, and frequency of complete recovery of blood flow after reopening of the MCAO in the pial arteries and veins.

Microaggregates appeared in the pial artery of most cats regardless of group, but the mean time to appearance in Group 2 (0.4±0.1 seconds) was significantly shorter (p<0.01) than that in Group 1 (121.2±32.2 seconds). On the other hand, adhesion of platelet aggregates to the arterial endothelium and subsequent formation of thrombi disturbing the blood flow were observed only in Group 1 (p<0.05, Fisher’s exact test). Stasis of arterial blood flow was noted in only one cat in Group 1 and in no cat in Group 2. After reopening of the MCAO, complete recovery of blood flow was never observed in Group 1 cats because of the remaining platelet thrombi in the pial arteries. In contrast, all cats of Group 2 showed full recovery of blood circulation, with complete disappearance of platelet microaggregates.

Microaggregates appeared in the pial veins in each group after MCAO, but the onset of appearance was significantly earlier in Group 2 than in Group 1 (p<0.01). Adhesion of platelet aggregates to the endothelium and formation of thrombi were observed in all cats of Group 1 but in only three of seven cats in Group 2. The incidence of this phenomenon was significantly higher in Group 1 than in Group 2 (p<0.05, Fisher’s exact test). In addition, the onset of adhesion of platelet aggregates to the endothelium was relatively earlier in Group 1 (117.9±21.8 seconds) than in Group 2 (493.3±97.5 seconds). Complete recovery of blood flow after reopening of the MCAO was observed in no cat in Group 1, but it was seen in four of seven cats in Group 2. The incidence of complete recovery of flow was significantly higher in Group 2 than in Group 1 (p<0.05, Fisher’s exact test).

Discussion

We clearly demonstrated that the stable prostacyclin analogue TRK-100 significantly reduces the hemorheologic derangement in the pial vessels following MCAO. As we reported previously, there is no doubt that the interaction of platelets with damaged endothelium plays a crucial role in initiating a vicious cycle of events that results in disturbance of the microcirculation after occlusion of a major cerebral artery. Our data showed that mild damage of the endothelium itself produced only minor changes in the microcirculation but that once the velocity of blood flow was decreased by MCAO, numerous platelet microaggregates became visible and began to adhere to the damaged endothelium, with the resultant formation of large thrombi.

In cats treated with TRK-100, abundant platelet microaggregates were produced at the site of the damaged endothelium, rather like fine wind-borne snowflakes, but they were continuously flushed away without forming large thrombi. This characteristic phenomenon can be explained as follows. It is known that prostacyclin inhibits platelet adhesion (platelet–collagen interaction) as well as platelet aggregation (platelet–platelet interaction); in addition, prostacyclin displays a disaggregating activity that tends to disperse platelet aggregates. TRK-100 shares all these properties. Thus, TRK-100 prevented platelets from sticking to the damaged endothelium so the damaged endothelium in our cats did not become covered with a platelet layer, and newly arriving platelets in the bloodstream continuously came into contact with the exposed surface of the vessel wall, to be activated at the site. Due to these properties of TRK-100, activated platelet aggregates could not accumulate on the damaged endothelium in cats in Group 2 so the appearance of platelet microaggregates in the bloodstream occurred significantly earlier after MCAO in Group 2 than in Group 1. We have encountered a similar effect with another prostacyclin analogue, iloprost (ZK 36374) (unpublished data), suggesting that the common biochemical property of these agents, increasing cAMP levels in the platelets by stimulating adenyl cyclase, may be closely associated with this effect.

As mentioned above, prostacyclin is an unstable metabolite of arachidonic acid and is synthesized...
mainly by endothelial cells. Prostacyclin is a potent vasodilating compound and an endogenous inhibitor of platelet aggregation. However, the control mechanism of prostacyclin synthesis is not yet well understood. It has been reported that platelet prosta-
glandin endoperoxides liberated during aggrega-
tion can become a substrate for endothelial pros-
tacyclin synthesis, providing a mechanism for
increased local prostacyclin production in response
to platelet aggregation in vivo.\textsuperscript{25,26} In addition, a
platelet-derived growth factor has been reported to
stimulate markedly the synthesis of prostacyclin in
the endothelium in vitro.\textsuperscript{27} However, our experi-
ments indicate that these mechanisms were insuffi-
ciently effective to prevent the hemorheologic
derangement observed in vivo after MCAO in
untreated cats.

It might be claimed that the damage produced by
the UV irradiation could have depressed the syn-
thesis of prostacyclin in the endothelium. The method of UV irradiation developed in our labora-

tory is highly useful for inducing selective endo-
thelial damage to the pial vessels in vivo,\textsuperscript{2} but the
damage to the endothelium is, at least morphologi-
cally, very slight and only small vacuolations can be
observed (Figures 3 and 4). During the UV irradiation
in our experiments platelet aggregates invariably
adhered to the endothelium, but after termina-
tion of the irradiation these adherent aggregates
delayly detached and were flushed away, and new
adhesion of platelet aggregates was never observed.
Based on these findings, there is no evidence to
suggest that the basic activity of prostacyclin syn-
theticase in the endothelium was impaired before
MCAO. However, examination of the biochemical
effects of UV irradiation on the endothelium is
warranted to correlate the morphologic alterations
with the metabolic changes, including prostacyclin
synthesis, in the endothelium.

The relatively weak effect of TRK-100 on the pial
vein compared with that on the pial artery might be
explained by the lower velocity of blood flow in the
vein, which resulted in a more intense platelet-
vessel wall interaction. Another possible explana-
tion is that the endothelial damage produced by the
UV irradiation may have been more severe in the
vein than in the artery.\textsuperscript{28} In fact, Group 1 cats
showed adhesion of the platelet aggregates to the
endothelium after MCAO significantly earlier in the
pial vein than in the pial artery ($p<0.01$). The concen-
tration gradient of TRK-100 between the
artery and the vein might offer another explanation.

After initiation of the TRK-100 infusion, the pial
arteries underwent a significant and progressive
dilatation with a slight decrease in MABP. Since we
have found that the autoregulatory mechanism of
the pial vessels in response to hypotension is impaired
after UV irradiation,\textsuperscript{29} these observations indicate
that TRK-100 dilates the pial arteries directly and
preferentially compared with the systemic vessels. As
illustrated in Figure 6, the diameter of the pial arteries
returned to the control level during the late phase of
MCAO in spite of a sustained reduction in local
perfusion pressure by MCAO, suggesting that TRK-
100 was useful not only in preventing the formation of
thrombi but also in maintaining the diameter of the
pial arteries under ischemic conditions.

Prostacyclin has been shown to be almost invari-
abley effective in experimental ischemic mod-
els,\textsuperscript{19,30-32} whereas controlled clinical trials have not
yielded positive conclusions.\textsuperscript{11,14-17} This discrep-
ancy between experimental and clinical studies could
be explained by the difficulty in maintaining an
effective blood concentration of prostacyclin in
patients due to its short half-life.\textsuperscript{8} In contrast,
TRK-100 has very stable chemical properties, and
its oral administration leads to an effective concen-
tration. Our results strongly support the rationale
for a double-blind clinical trial of TRK-100, which is
currently being undertaken in Japan.

Note added in proof: TRK-100 has recently
been given the international nonproprietary name
bepraprost with the approval of the World Health
Organization.

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